In claims 29, 30 and 40, line 3, after "a" and before "gene", please insert --heterologous--.

Remarks

I. Status of the Claims

Claims 1-40 are pending in the present application. Claims 1, 9, 19, 29, 30, and 40 have been amended to recite that the tissue-specific regulatory dequence is heterologous to the coding sequence to which it is operably linked. Support for the amendment can be found, *inter alia*, on page 6, line 29.

II. The Rejections

A. Rejection under 35 U.S.C. § 112, first paragraph

On page 2 of the Office Action, claims 1-40 have been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that these claims are not enabled. Applicants respectfully traverse the rejection.

First, the Examiner asserts that no tissue-specific vector has been enabled because the construction of the specific plasmids is described only by incorporation by reference. Applicants respectfully disagree, because it is not the specific plasmid and no other that enables the production of a tissue-specific vector. Any of the various known promoters could have been

operably linked to the coding region of a gene essential for replication (from which the promoter has been removed) based on published sequences and/or restriction maps. Assays for functional promoters (i.e., transcription assays) were routine in the art.

Applicants respectfully point out that these are working examples only, and are not essential for practicing the claimed invention. If the Examiner has reason to doubt that one of ordinary skill in the art, provided with the concept which the Applicants have provided, could not have operably linked any of the known promoters to genes essential for replication, specific evidence or scientific reasons should be presented to support this position. Since this has not been done, Applicants respectfully submit that a *prima facie* case of non-enablement on these grounds has not been established.

Further, since the material cited by the Examiner is not essential material to practice the claimed invention, the issue of the incorporation of essential material by reference to a pending application is moot. Applicants therefore submit that it is not required to amend the disclosure to include the material incorporated by reference.

The objection is also made that no information has been provided regarding Addl 327. Again, enablement in the present case does not depend upon the preparation of a specific plasmid, since the isolation and assaying of promoters with various kinds of coding sequences was routine in the art at the time the application was filed. Given the general principles disclosed in the specification, the claimed vector could have been built from any virus in which the genes essential for replication had been characterized, with any of the various known promoters.

Accordingly, unless evidence is provided to the contrary showing that the invention was not enabled except with this vector, Applicants respectfully submit that a *prima facie* case of non-enablement on these grounds has not been made.

Applicants respectfully assert that a *prima facie* case of nonenablement has not been established on any grounds further presented by the Examiner. The initial burden is on the Patent Office to provide convincing evidence supporting the conclusion that the person of ordinary skill would not have been enabled to practice the claimed invention throughout the entire scope of the claim. See, *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). As discussed below, in the case of several aspects of the Examiner's rationale, insufficient or no evidence has been supplied showing how the person of ordinary skill in the art could not have made or used the invention without an undue burden of experimentation.

Undue experimentation is a conclusion that is reached by weighing many factual considerations. These considerations have been enumerated in *Ex parte Forman*, 230 USPQ 546, 547 (PTO Bd. App. 1986). These include (i) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

A determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. The test is not merely quantitative since a considerable amount of

experimentation is permissible, if routine or if the specification in question provides sufficient guidance with respect to the direction in which the experimentation should proceed in order to enable one of ordinary skill in the art to practice the claimed invention. The emphasis is not on the term "experimentation" but on the term "undue".

The case law shows that the scope of enablement is directly proportional to the pioneering nature of the invention. Thus, an invention made in a crowded art may be limited commensurately in scope while a pioneering invention deserves commensurately broad scope.

Applicants respectfully submit that the rejection has been based *entirely* on consideration of only one of the Forman factors: the *predictability* factor. Further, Applicants respectfully submit that insufficient evidence has been offered to support the conclusion that the invention is sufficiently unpredictable that it is not enabled to any degree. Accordingly, as discussed below, Applicants respectfully disagree with the rationale for the rejection and submit that a *prima facie* case of nonenablement has not been established.

On page 4 of the Office Action, a general objection is raised that the field of "gene regulation is recognized as highly experimental and unpredictable." The standard for enablement in the patent law is "*reasonable* predictability." Applicants respectfully submit that the objection has not been specifically applied to the case at hand. There are various aspects of gene regulation that are routine and reasonably predictable.

The Examiner has presented no reason to explain how the attachment of a promoter to a heterologous coding sequence is not reasonably predictable. The Examiner has also failed to explain why one would not expect vector replication to occur when a heterologous promoter is operably linked to a coding sequence for a gene required for replication. Accordingly, no evidence or scientific reason has been presented that would support the position that it is not reasonably predictable that a promoter operably linked to such a coding sequence could not have been made or used without an undue burden of experimentation.

As evidence supporting the rejection, Dillon W., *Trends Biotechnol. 11*:167-173 (1993), is cited. This reference is relied upon to show that the field of gene regulation "is recognized as highly experimental and unpredictable." However, the standard is reasonable predictability. The Examiner asserts that the reference reviews attempts to use tissue-specific promoters in gene therapy, and concludes that the reference shows that "therapeutic genes can be delivered into target tissues with varying efficiency, but . . . also demonstrate[s] that controlled expression of the gene remains a problem and that a considerable degree of optimization will be required in order to achieve therapeutic levels of gene expression in the appropriate tissues."

Applicants point out that this reference demonstrates that therapeutic genes can in fact be delivered into target tissues with varying degrees of efficiency. Nevertheless, it states that they *can* be delivered.

The reference asserts that controlled and/or reproducible expression remains a problem. Applicants point out, however, that the reference relates to gene therapy in which a specific level

of a gene is required for therapeutic results in treating a cell. The premise of Dillon is that for gene therapy to succeed as a strategy for treating human disease, it is necessary for a therapeutically useful gene to be delivered efficiently to the cells of the appropriate tissue and once it has been delivered, to be expressed at a level sufficient to achieve the desired effect. This is not germane to the claimed invention. In the instant case, only replication of the vector is required.

Claims 1-40 are not directed to controlled expression. Claim 1 is directed to a vector capable of tissue-specific replication comprising a transcriptional regulatory sequence operably linked to the coding region of a gene essential for replication of the vector. Claim 9 is directed to a method for distributing a polynucleotide in a tissue *in vivo* by introducing the vector above. Claim 19 is directed to a cell containing the vector. Claim 29 is directed to a method for making the vector. Claim 30 is directed to a cell containing a vector virion. Claim 40 is directed to a method for reproducing the virion. Thus, none of the claims is even related to levels of expression for gene therapy. They are related to vector replication.

Thus, Applicants respectfully submit that the reference is not relevant to the claims. If it is, the Examiner has not explained what the relevance is.

On page 5 of the Office Action, as further evidence, the Examiner submits a report and recommendations of an *ad hoc* committee designed to assess the NIH investment in research on gene therapy. The arguments discussed above apply to this reference.

The Examiner cites this reference specifically for the teaching that "determining the appropriate dosage levels for viral vectors presents another major challenge." Office Action, page 5. It is then concluded that because finding the appropriate dosage is another major challenge, the burden of experimentation would be undue. No evidence specifically related to the claims is presented to support the conclusion.

The Examiner further objects that there is not sufficient "guidance from the specification or the art concerning the details of promoter and enhancer gene sequences." The Examiner then concludes that the person of ordinary skill in the art would be unable to make the claimed vectors with a reasonable expectation of success without undue experimentation. Again, no evidence is presented showing this to be the case.

The specification need not teach what is routine in the art. Coding sequences and open reading frames are routinely isolated and operably linked to promoters to obtain hybrid transcription cassettes. The Applicants need not present specific promoter or enhancer sequences since these sequences are well-known to those of ordinary skill in the art and it is understood and routine to operatively link these sequences to an appropriate coding sequence. Accordingly, there is no reason why an appropriate coding sequence in a virus could not be operably linked to such a heterologous transcriptional control sequence. In any event, the Examiner has failed to specifically point out why this is not so or should not be so in the present case.

On pages 6 and 7, the Examiner provides further "evidence" to support the conclusion. However, again, this evidence is general and not specifically directed to the claimed constructs.

The Examiner fails to explain how these references specifically relate to the claimed constructs and how these generalizations should apply to the constructs at hand. The quotations set forth by the Examiner are generalizations at best. If the rejection is maintained, the Applicants respectfully request that the Examiner relate these problems to the claimed constructs. Where is the text specifically relevant to the claimed vectors, i.e., where *replication* is driven by heterologous promoter?

On page 7, Pennisi (*Science 274:342-343* (1996)) is cited. The Examiner takes the position that the claimed vectors would not be effective therapeutic agents because the patient's immune system would prevent the establishment of an effective infection. Applicants respectfully disagree as follows.

In the introduction, Shingu (Appendix) briefly discusses anticancer therapy in humans citing it as "one of the most difficult medical problems." It is indicated that of the biological agents that have been used for anticancer therapy "none . . . is *completely* effective" (emphasis added). The problems cited are non-specificity of the anticancer agent and severe side-effects. Shingu indicates that the use of viruses for therapy of malignant tumors is one of the strategies that employs biologically active agents, but no more is said on the subject. The introduction does indicate, however, that "some bovine enteroviruses have an oncolytic effect on solid tumors and prolong the life span of mice bearing human carcinoma cells." Shingu, page 2031, column 1.

In the study reported, bovine enterovirus was shown to be effective against a rabbit model of ATL, a disease caused by HTLV-1 in which patients usually die within about 4 months.

Shingu, page 2031, column 2. Thus, the cited study showed the effectiveness of viral treatment, prompting the author to indicate that the results suggested "that bovine enterovirus might be used clinically to prolong the life span of ATL patients".

The therapeutic effects of viral infection were examined in rabbits used as a model for ATL. Use of the virus increased the life span from 10-22 days to 4 months. Thus, the reference submitted by the Examiner supports the efficacy of viral treatment. The authors conclude "in this study, bovine enterovirus is effective in the treatment of ATL in experimentally treated animals." Shingu, page 2033, column 1. It is also stated that "a distinct therapeutic effect was observed." Shingu, page 2033, column 2.

Thus, the reference discusses potential problems which need to be addressed for maximally effective use of virus in cancer treatment. But this caveat in no way provides or supports the position that undue experimentation would have been involved to overcome these potential problems. In fact, the text goes on to state "although these problems remain, virus injected intraperitoneally can be effective in the treatment of end-stage ATL patients in which it reduces the number of ascites cells disseminating from infiltrated organs, such as the liver, lung and spleen. It is possible that this route of administration will prolong the life span of the ATL patient."

Accordingly, Applicants respectfully submit that the evidence submitted by the Examiner does not establish a *prima facie* case of nonenablement based on the rationale that the immune system would neutralize virus before it reached the target.

Nevertheless, Applicants discuss below why viral therapy would be expected to be prevented by the immune system. First, 50% of the human population does not have neutralizing antibodies against adenovirus. The ones that do, rarely have neutralizing titers so high as to inactivate a clinically-effective dose (for example 10^9 PFUs) administered in a clinically-effective manner, such as directly into a tumor. Thus, 50% of the population could be treated even if neutralization were a problem. However, even if the neutralizing antibody titer were a rare 1/50,000, then at most much *less than half* of the dose provided to the tumor would be inactivated.

It is also highly unlikely that the milieu of a tumor would contain sufficiently high levels of neutralizing antibody to prevent clinical effect. In fact, a clinical trial involving replication competent adenovirus (Ad5-derived) in head and neck cancer patients was discussed recently at the Biological Society meeting held in Washington, DC. No diminution in transduction was described after four repeated intratumoral administrations. In addition, replication was also observed. Other clinical trials undertaken with adenoviral vectors administered *in vivo* in humans show that the effect of neutralizing antibodies can be countered by sufficiently routine increase in viral load, i.e., by out-titering any potentially problematic neutralizing antibodies.

Further, a T cell response elicited against tumor cells harboring virus may actually help to elicit a secondary immune response against the tumor. It is well-known that viral oncolysis helps to elicit a strong immune response. The results of a clinical trial at the National Cancer Institute utilizing a replication competent vaccinia virus expressing the tumor antigen CEA showed that this combination elicited a T cell response against tumor cells carrying CEA. This

trial showed that a viral vector can efficiently infect a tumor cell without being inactivated by the immune system prior to reaching the target cells.

Applicants also point out that it is well-known that patients having prior exposure to adenovirus can die of adenoviral infection (for example AIDS patients). Thus, replication can occur in humans to a significant extent even in the presence of prior antibodies against adenovirus.

The above evidence shows that interference by the immune system, if it occurs at all, can be overcome by the implementation of routine empirical procedures. In any event, the fact that viral therapy is a viable ongoing form of therapy shows that this form of therapy is not fatally flawed in the early stages by inactivation of the vector itself.

Further, the viral vector need not reach every tumor cell. Since the vector would be replicating only in tumor cells, it need only transduce a small portion of them. For example, as taught in Applicants specification, an intra-tumoral delivery of 2×10^9 PFUs is sufficient for efficacy in human hepatocellular tumors. This is because of the multiplication and spread of infectious virions after replication in the initially infected tumor cells.

Applicants also point out that intraperitoneal, intravenous, intratumoral, and hepatic artery injections of adenoviral vectors would be reasonably expected to provide efficient transduction of tumor cells. This has been well-documented in the literature. Shingu *et al.* shows this. While the reference states that transduction of the tumor may be a problem, the

reference actually shows that a replicating bovine enterovirus has efficacy against AML in an immunocompetent animal. A tumor specific replication competent adenovirus would be expected to be even more effective because the bovine enterovirus is not even tumor-specific.

In view of the above discussion, Applicants respectfully submit that a *prima facie* case of nonenablement has not been made because sufficient evidence has not been presented supporting the Examiner's position. Even if this evidence has been presented, Applicants have shown sufficient evidence to overcome the *prima facie* case and show why an undue burden of experimentation would not have been required to practice the claimed invention. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

B. Rejection under 35 U.S.C. § 102

1. Ringold

On page 8 of the Office Action, claims 1, 2, 10, 19, 20, 22, 29-31, 33 and 40 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Ringold *et al.* (*Proc. Natl. Acad. Sci. USA 74:*2879-2883 (1977), here in "Ringold"). Applicants respectfully traverse the rejection.

Ringold teaches that glucocorticoid hormones increase the amount of mouse mammary tumor virus RNA in a cultured cell line from a mouse mammary carcinoma and hepatoma. It reports that the steroids have no effect on the amount of RNA in a lymphoma cell line, which produces large basal amounts of the RNA.

First, there is no certainty that the effect is achieved at the transcriptional level. The reference clearly states that it cannot rule out that the effect is due to the inhibition of RNA degradation. Thus, it is not a certainty that the effect is achieved by way of a transcriptional regulatory sequence. Further, there is no evidence that if the effect occurs by way of a transcriptional regulatory sequence, that the transcriptional regulatory sequence is tissue-specific, i.e., that it responds to a factor present in one cell type but not in another. Note that transcription occurs in all of the cell types studied. Third, the viral sequences are integrated into the genome. Thus, even if the effect occurred via a transcriptional regulatory sequence, it is not known whether the transcriptional regulatory sequence is found in the integrated viral sequence. Even less is it known whether such a transcriptional regulatory sequence is operably linked to a gene in the viral sequence. The effect could occur on cellular sequences with, for example, transactivation of promoter sequences in the vector.

Applicants point out that anticipation cannot legally be based upon speculation or probability but must be based on certainty. Because the reference does not unequivocably show that the effects are due to a transcriptional regulatory sequence in the vector, Applicants respectfully submit that the reference does not anticipate the claims.

As to claims 2, 10, 20 and 31, Ringold does not show tissue-specific promoters or enhancers. As to claim 29, Ringold does not teach recovery of the vector. As to claim 40, Ringold does not teach recovery of a virion. As to claim 30, Ringold does not teach replication of the virion. Ringold merely assays RNA synthesis.

For all these reasons, Applicants respectfully submit that Ringold does not anticipate the claims. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

2. Gunzburg

On page 9 of the Office Action, claims 8, 27 and 38 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Gunzburg *et al.* (*Virology 155*:236-248 (1986), herein "Gunzburg"). Applicants respectfully traverse the rejection.

Gunzburg is relied upon as disclosing a viral vector based on mouse mammary tumor virus in which the *env* coding region has been removed and replaced with the *neo* coding region. This vector does not meet the limitations of the claims because the *neo* gene is not essential for replication. To point out the difference, the claims have been amended to clearly recite that the coding region of the gene essential for replication is operably linked to a heterologous transcriptional regulatory sequence.

In view of the amendment of the claims, Applicants respectfully submit that the claims are clearly distinguished over Gunzburg. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

3. McCormick

On page 10 of the Office Action, claims 4-7, 23-26 and 34-37 have been rejected under 35 U.S.C. § 102(b) as being anticipated by McCormick (WO 94/18992). Applicants respectfully traverse the rejection.

The Examiner relies on McCormick as disclosing an adenovirus "in which the E1A coding region is under the control of a tissue-specific regulatory sequence which allows replication only in tissues lacking a functional p53 protein." This is incorrect. The E1A gene product is not under the control of a tissue-specific regulatory sequence. The E1A gene product binds to p53, a protein involved in cell cycle regulation. This disregulation allows uncontrolled cell division and along with cell division, replication of the virus. Tissues lacking a functional p53 protein thus do not even require an E1A protein to inactivate the p53 protein since p53 protein is in fact is functionally absent from the cell. Accordingly, Applicants respectfully submit that the Examiner has misapprehended the relationship between E1A and p53. The Examiner appears to take the position that p53 regulates transcription from the E1A coding region by virtue of a tissue-specific regulatory sequence. As explained above, however, this is not the case.

Accordingly, Applicants respectfully submit that the grounds for the rejection have been addressed and the rejection overcome. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

C. Rejection under 35 U.S.C. § 103

On page 11 of the Office Action, claim 28 has been rejected under 35 U.S. C. § 103(a) as being unpatentable over Gunzburg in view of Culver (*Science 256*:1550-1552 (1992), herein "Culver"). Applicants respectfully traverse the rejection.

Gunzburg does not suggest operably linking a heterologous transcriptional regulatory sequence to a gene essential for replication. The Examiner appears to take the position that it would have been obvious to substitute the anti-tumor gene of Culver for the *neo* gene of Gunzburg. Even is this is the case, however, the reference does not suggest using a heterologous promoter to drive a gene essential for replication. Since neither the *neo* gene nor the anti-tumor gene provides a coding region for a gene product necessary for replication, neither Gunzburg nor Culver suggests the claimed invention. Further, for these reasons, there would have been no motivation to combine Culver and Gunzburg. Even if the references had been combined, the claimed invention would not have been obtained.

For the above reasons, Applicants respectfully submit that the grounds of rejection have been addressed and the rejection overcome. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

III. Conclusion

Applicants respectfully submit that all of the grounds for rejection have been addressed and the rejections overcome. Applicants believe, therefore, that the application is in condition for immediate allowance. If the Examiner believes that an interview regarding the issues herein would be helpful for expediting prosecution, he is invited to contact Applicants' attorney by telephone at 202-371-2630.

Respectfully submitted,

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APPENDIX

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Therapeutic effects of bovine enterovirus infection on rabbits with experimentally induced adult T cell leukaemia

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A bovine enterovirus, MZ-468, showed cytopathic effects on cell line F-647a, which was established by coculture of human T cell lymphotropic virus type 1-transformed MT-2 cells and X-irradiated rabbit lymphocytes. Microcalorimetric assay showed that residual, viable, MZ-468-infected F-647a cells produced less heat than non-infected cells. The therapeutic effects of MZ-468 infection were examined in rabbits in which adult T cell-like leukaemia (ATL) had been induced by inoculation of F-647a cells (1×10^8 cells). Six newborn rabbits were separated into three groups: group A was inoculated with F-647a cells only; group B was treated with MZ-468 at the time of inoculation with cells; group C was treated with the same amount of virus 24 h after the inoculation with cells and then once every 4 days. Both of the animals in group A and one in group C died 10 and 11 days, and 22 days, respectively, after the inoculation with cells. Both rabbits in group B and one in group C survived for more than 4 months. The rabbits that died were examined pathologically; leukaemic infiltrations were found in the lungs of the group B rabbits, and in the lungs, spleens and livers of both group A rabbits. Two identical experiments produced almost the same findings. These results suggest that bovine enterovirus might be used clinically to prolong the life-span of ATL patients.

Anticancer therapy in humans is one of the most difficult medical problems; a great number of chemical and biological agents have been used for this purpose, none of which is completely effective. The problems involved include the non-specificity of the action of the anticancer agents, which is accompanied by severe side-effects. The use of viruses for therapy of malignant tumours is one of the strategies which employ biologically active agents (Casto & Hammon, 1970; Hunter-Craig *et al.*, 1970; Taylor *et al.*, 1971). Our laboratory has tried to identify those viruses which show strong oncolytic effects and minimal side-effects (Shingu, 1976, 1981; Kaneko *et al.*, 1979), and we have found that bovine enteroviruses lytically infect mouse fibroblast cells only when the cells are transformed with simian virus 40 (Shingu, 1976). We have also found that some bovine enteroviruses have an oncolytic effect on solid tumours and prolong the life-span of mice bearing human carcinoma cells (Fujimaru, 1978).

Seto *et al.* (1988) recently have succeeded in inducing a disease closely resembling an acute type of adult T cell-like leukaemia (ATL) in rabbits by inoculating syngeneic human T cell lymphotropic virus type 1 (HTLV-1)-transformed cells into newborn rabbits. The HTLV-1-transformed T cell line, F-647a, was established from a 2-month-old female (B/J × Chbb:HM) F1 rabbit, which had been infected with the virus neonatally. In brief, peripheral blood lymphocytes were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), in the absence of T cell growth factor. The established cell line had the karyotype of a normal female rabbit, and was positive for the HTLV-1 p19 antigen, the pan T cell marker antigen and the Ia antigen, which were detectable using monoclonal antibodies. The F-647a cell line was able to kill newborn rabbits within 7 days when a dose of 1×10^8 cells was inoculated intraperitoneally. In this study we demonstrated that bovine enterovirus has effects against a rabbit model of ATL, a disease caused by HTLV-1 in which patients usually die within about 4 months.

A bovine enterovirus of type 1 subtype 1, MZ-468, was isolated from calf faeces (Tamada, 1977; Urakawa & Shingu, 1987). The virus was propagated in HeLa cells, purified by equilibrium centrifugation in a CsCl gradient and plaque assayed in Wilms tumour cells originally established in this laboratory (Shingu & Chibana, 1981). F-647a cells (Seto *et al.*, 1988) were kindly provided by Professor A Seto, Siga University School of Medicine, Japan, and passaged in RPMI 1640 medium (Nissui) containing 10% FCS (Gibco).

MZ-468 (5·0 p.f.u./cell) was inoculated and adsorbed onto suspended F-647a cells (4×10^4) for 1 h at 37 °C in plastic dishes (Falcon), and incubated at 37 °C under 5% CO_2. Virus propagation was examined every 6 to 12 h by

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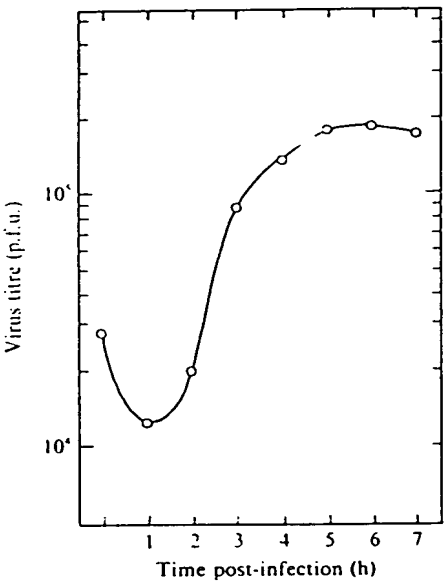

Fig. 1. One-step growth of MZ-468 in MZ-647a cells. F-468 (m.o.i. 5) was adsorbed onto 4×10^4 cells in 1 ml of RPMI 1640 containing 10% FCS and incubated at 37 °C in the presence of 5% CO_2. The virus titre at each time point was determined by plaque assay.

plaque assay in monolayers of Wilms tumour cells. The average virus titre was determined from four or five cultures and MZ-468 replication in F-647a cells was confirmed using a one-step growth curve (Fig. 1).

To determine the oncolytic activity of MZ-468 against F-647a cells in tissue culture, two different techniques were chosen to measure cell activity: measurement of heat production, and the number of cells present after 1, 2 and 3 days of cultivation.

A flow-type microcalorimeter (thermoactive cell analyser ESCO-3000, Denshi Kagaku) was used to measure heat production. Washing medium (1·8 ml), guide medium (1·8 ml) and sample medium (0·6 ml) containing 2×10^6 suspended cells were added, in that order, to the microcalorimeter. Cell suspension (0·6 ml) was transferred into the microcalorimetric tube which was then introduced into the reaction vessel and heat production was recorded over a period of 15 min. The same medium used for cell culture was used for the measurement of heat production and oxygen consumption. The heat produced by MZ-468-infected (m.o.i. 13) or uninfected F-647a cells was measured after 24, 48 and 72 h of culture at 37 °C. Heat production, i.e. thermal power, was expressed as $\mu W/10^6$ cells, and the result presented as a percentage of the rate of heat production.

Cells were counted in a Bürker chamber. Cell viability was checked by the trypan blue exclusion test during culture and before microcalorimetry.

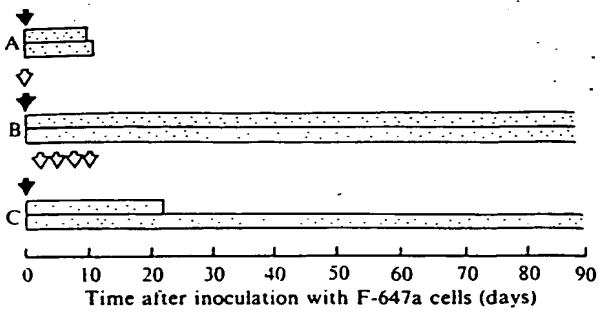

Fig. 2. Schematic representation of procedures and the results of an experiment in which 3-day-old rabbits with a form of ATL were treated with a bovine enterovirus. Solid arrow, inoculation with 10^8 F-647a cells; open arrow, inoculation with $2{\cdot}8 \times 10^7$ p.f.u. MZ-468. Length of bars indicates the life-span of each animal.

Table 1. *Effect of virus infection on viability of and heat production by viable F-647a cells*

Time post-infection (h)	Native F-647a		Virus-infected F-647a	
	Viable cells ($\times 10^{-6}$)	μW (%)	Viable cells ($\times 10^{-6}$)	μW (%)
0	4·3			
24	7·2	100	1·2 (16·7)*	11·3
48	7·1	100	1·0 (14·1)	6·3
72	16·5	100	0·3 (1·8)	1·6

* Figures in parenthesis represent the number of viable cells expressed as a percentage of the number of viable, uninfected cells.

Infection of F-647a cells in RPMI 1640 by MZ-468 reduced the viability of and production of heat by the residual viable cells, as shown in Table 1. A good correlation existed between the number of cells and the results of the microcalorimetry experiments, indicating that bovine enterovirus can kill F-647a cells.

Six newborn New Zealand white rabbits (3 days old) from one litter were separated into three groups which were treated as shown schematically in Fig. 2. Group A was injected intraperitoneally with cells only (1×10^8); group B was injected with the same number of cells and $2{\cdot}8 \times 10^7$ p.f.u. MZ-468; group C was injected with the same number of cells, and 24 h later virus ($2{\cdot}8 \times 10^7$ p.f.u.) was injected intraperitoneally and this was repeated once every 4 days. All the rabbits were allowed to suckle milk from their mother in a special, separate box.

Results of this experiment are shown schematically in Fig. 2. The group A animals (receiving no treatment with MZ-468) died 10 and 11 days after inoculation with cells. Pathologically, leukaemic infiltrations were found in the lungs, spleens and livers. In contrast, both group B

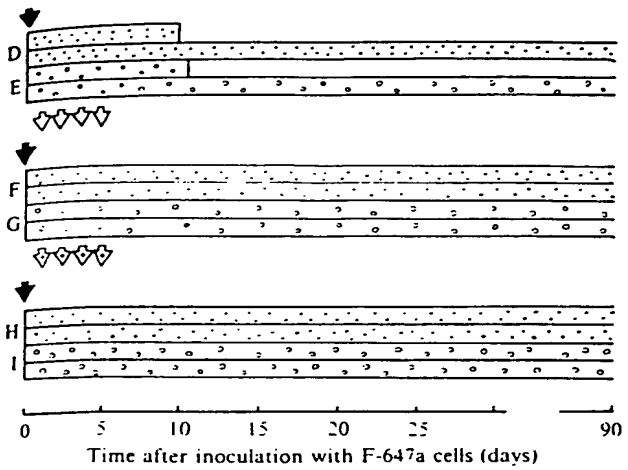

Fig. 3. Twelve newborn rabbits were inoculated with HTLV-1-transformed F-647a cells and divided into groups D, F and H (inoculated with 1×10^8 cells), and E, G and I (inoculated with 6×10^7 cells). Groups D and E were control, untreated groups; two rabbits, one from each group, died 10 and 11 days after inoculation. Groups F and G were treated with MZ-468. Groups H and I were treated with F-647a cells onto which MZ-468 had adsorbed. Groups F, G, H and I were inoculated with MZ-468 or cells adsorbed with virus 1 day after inoculation with F-647a cells and once daily for 4 days. These rabbits were cured without showing any side-effects over a 90 day period. Solid arrows, inoculation with F-647a cells; open arrows, inoculation with $2{\cdot}2 \times 10^6$ p.f.u. MZ-468 or $4{\cdot}4 \times 10^5$ F-647a cells onto which $2{\cdot}2 \times 10^6$ p.f.u. MZ-468 had been adsorbed. The length of the bars indicates the life-span of each animal.

animals (which were simultaneously treated with MZ-468 and the ATL-derived cells) survived for more than 4 months. One of the animals from group C (which was treated 1 day after inoculation with cells) died showing leukaemic infiltration, but the other rabbit survived for longer than 4 months. Two other similar experiments produced almost the same results and pathological findings (data shown in Fig. 3). In this study, bovine enterovirus is effective in the treatment of ATL in experimentally treated animals.

The host range of a virus is generally restricted as to species and this restriction is dependent on the existence of cellular receptors. Bovine enteroviruses do not infect primary cultured human cells, but do infect malignant human cell lines (Shingu, 1976). A possible mechanism for this change in sensitivity may be that the cell membrane changes molecularly during transformation, a phenomenon derived from derepression and amplification of proto-oncogenes, as found for c-*erbB*-2 (Semba *et al.*, 1985). Thus, expression of cell proto-oncogenes, especially those of the receptor type may produce targets for the adsorption of virus. Another mechanism for a viral oncolytic effect involves unmasking or xenozenization (Sissons & Oldstone, 1985) of membrane molecules, which might also act as virus receptors. These properties will enable us to utilize the bovine enterovirus as an anticancer biological agent which does not damage normal organs, because only malignant cells will be infected lytically. In agreement with this, many types of enterovirus have been examined experimentally for anticancer activity *in vitro* and *in vivo*, and a number have shown remarkable oncolytic effects against solid tumour cells (Imamura & Shingu, 1985). The life-spans of mice transplanted with various human malignant cell lines have been extended by injections of enteroviruses (Ishida, 1984).

We have used a variety of techniques to identify the bovine enterovirus receptor on tumour cells. Receptors for the bovine enterovirus are present on the surface of human adenocarcinoma cell lines, some haematopoietic cell lines of the B cell lineage and activated T cell lines. Anti-HLA-DR monoclonal serum was found to block binding of the oncolytic bovine enterovirus to Raji (B cell lineage, Burkitt's lymphoma origin) or MT-2 cells (T cell lineage, ATL origin), under conditions in which normal mouse serum did not block virus binding. The receptor for the oncolytic bovine enterovirus on the tumour cell is closely associated with the HLA-DR antigen (Shingu, 1989). In this study rabbit models of ATL were used as targets for MZ-468, and a distinct therapeutic effect was observed. This suggests that MZ-468 might be used clinically for treatment of ATL. However, practical administration will necessitate a solution to the potential problems of an anaphylactic reaction and antibody production against the virus after repeated injection. Another problem to be overcome is the identification of a means of virus administration by which it efficiently reaches its targets. Although these problems remain, virus injected intraperitoneally can be effective in the treatment of end-stage ATL patients, in which it reduces the number of ascites cells disseminating from infiltrated organs, such as the liver, lung and spleen. It is possible that this route of administration will prolong the life-span of the ATL patient.

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